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Feline infectious peritonitis: Role of the feline coronavirus 3c gene in intestinal tropism and pathogenicity based upon isolates from resident and adopted shelter cats

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ABSTRACT

Feline infectious peritonitis virus (FIPV) was presumed to arise from mutations in the 3c of a ubiquitous and largely nonpathogenic feline enteric coronavirus (FECV). However, a recent study found that one-third of FIPV isolates have an intact 3c and suggested that it is not solely involved in FIP but is essential for intestinal replication. In order to confirm these assumptions, 27 fecal and 32 FIP coronavirus isolates were obtained from resident or adopted cats from a large metropolitan shelter during 2008–2009 and their 3a–c, E, and M genes sequenced. Forty percent of coronavirus isolates from FIP tissues had an intact 3c gene, while 60% had mutations that truncated the gene product. The 3c genes of fecal isolates from healthy cats were always intact. Coronavirus from FIP diseased tissues consistently induced FIP when given either oronasally or intraperitoneally (i.p.), regardless of the functional status of their 3c genes, thus confirming them to be FIPVs. In contrast, fecal isolates from healthy cats were infectious following oronasal infection and shed at high levels in feces without causing disease, as expected for FECVs. Only one in three cats shed FECV in the feces following i.p. infection, indicating that FECVs can replicate systemically, but with difficulty. FIPVs having a mutated 3c were not shed in the feces following either oronasal or i.p. inoculation, while FIPVs with intact 3c genes were shed in the feces following oronasal but not i.p. inoculation. Therefore, an intact 3c appears to be essential for intestinal replication. Although FIPVs with an intact 3c were shed in the feces following oronasal inoculation, fecal virus from these cats was not infectious for other cats. Attempts to identify potential FIP mutations in the 3a, 3b, E, and M were negative. However, the 3c gene of FIPVs, even though appearing intact, contained many more non-synonymous amino acid changes in the 3' one-third of the 3c protein than FECVs. An attempt to trace FIPV isolates back to enteric strains existing in the shelter was only partially successful due to the large region over which shelter cats and kittens originated, housing conditions prior to acquisition, and rapid movement through the shelter. No evidence could be found to support a recent theory that FIPVs and FECVs are genetically distinct.

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1. Introduction

The internal mutation theory on the pathogenesis of FIP asserts that FIPVs arise by mutation during infection with FECVs (Poland et al., 1996; Vennema et al., 1998). Although subsequently challenged (Dye and Siddel, 2007; Brown et al., 2009), the internal

mutation theory has been validated by several recent studies (Chang et al., 2010, 2011; Pedersen et al., 2009). FECV mutants that attain the FIP biotype have gained tropism for macrophages (reviewed Pedersen, 2009). This altered tropism allows the virus to stray from its normal host cell (mature intestinal epithelium) and become a systemic pathogen of macrophages, a cell type that is at the core of both innate and adaptive immunity. The type and strength of immune responses to this macrophage infection is thought to dictate whether or not a cat becomes diseased, and if diseased, the form it takes (reviewed Pedersen, 2009).

Mutations that increase macrophage tropism were originally thought to reside solely in the 3c gene, to work by altering the size of the gene product, and to be unique to each cat dying of FIP

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(Vennema et al., 1998; Pedersen et al., 2009). The 3c is one of 11 genes in the feline coronavirus genome and it is uncertain whether it is an integral part of the virion and its exact function in the virus life-cycle. It encodes a triple-spanning membrane protein that is similar in hydrophobic profile, but not sequence, to the M protein (Oostra et al., 2006). Although the basic role of internal mutation endures, the essential role of 3c mutations in the FECV to FIPV conversion has been questioned. It is now apparent that one-third or more of FIPV isolates have 3c genes that are intact, which is the case for all FECVs (Chang et al., 2010). Based on their findings, Chang and colleagues (2010) concluded that whereas an intact 3c is essential for intestinal replication, functional mutations in 3c are not essential for increased macrophage tropism and FIP. However, neither of these conclusions was tested by experimental cat inoculation studies. Rather, the disease potential (biotype) of their coronavirus isolates was defined by their origins, i.e., FECV if they were found in feces and FIPVs if they were isolated from diseased tissues. The implications of these findings go beyond the nature of the FIPV mutation(s). If FIPVs with intact 3c can replicate in the intestine, they may also transmit cat-to-cat. This is counter to the current corollary that FIPV is rarely if ever transmitted horizontally and that cats with FIP are therefore not risks for other cats (reviewed by Pedersen, 2009; Pedersen et al., 2009).

The present research is an extension of the work of Chang and colleagues (2010) in determining the role of 3c for intestinal replication of both FIPVs and FECVs. The FECV and FIPV isolates used in this study were obtained from a large metropolitan shelter in San Francisco that receives kittens and adult cats from the Bay Area. The shelter staff educates owners about the health problems that may occur in their new pet, with emphasis on serious diseases such as FIP. Owners are also encouraged to seek veterinary care from the shelter staff or private veterinarians if FIP or signs of FIP occur. As a result of these practices, many of the FIP cases among adoptees come to the attention of the shelter staff. Therefore, this collaboration yielded a large number of both FIPV and FECV isolates. The E, M, 3a–c genes were then sequenced and used to categorize the various isolates for their 3c gene status. A subset of these genetically characterized isolates were then used to infect laboratory cats both oronasally and by the i.p. route to determine their disease potential and ability to replicate in the intestine. Additional studies were also conducted on isolates obtained after animal passage.

2. Methods and materials

2.1. Field material

Cats sampled in this study were from the San Francisco Society for the Prevention of Cruelty to Animals (SF SPCA) shelter. This shelter intakes approximately 3000 cats a year, about 50% of which are kittens. Cats and kittens are obtained from a local animal control shelter, from local citizens, or from a number of regional orphan and abandoned kitten foster/rescue organizations. Older kittens are often taken directly into the shelter, while kittens received at 4–5 weeks of age are immediately transferred to local volunteer foster caregivers. Foster caregivers house one to several litters of kittens at a time until they attain the necessary age and weight for spaying or castration (9–12 weeks and 0.9–1.0 kg), at which time they are returned to the SF SPCA shelter. There is a biphasic peak in kitten numbers in the shelter with the first peak in spring, a decrease in August and September and a second wave around October through December. Kitten numbers decrease to very low levels during the winter (January to March/early April). This seasonal rush causes crowding at all levels of the kitten relinquishment/adoption cycle and the incidence of coronavirus infection correlates with

Table 1
Source of inocula used in experiments.

Inoculum	Original source (Cat #)
FECV-1	Feces of SFS-25, 26, 27, 31
FCov-1	Feces of SFS-05
i3c-1	Omentum of SFS-01, 04, 10, 16
i3c-2	Omentum of experimental cats 09-017, 09-024, 09-027 that had been infected with i3c-1
m3c-1	Omentum of SFS-02, 03, 08, 20
m3c-2	Omentum of experimental cat 09-022 that had been infected with m3c-1
ddFIPV	Feces from experimental cats infected with m3c-1

population density, i.e., low in winter and high in late spring and early summer.

Forty adoptees were known to have died of FIP between June and December 2008 and samples of diseased tissues were obtained at necropsy by either private veterinarians or the shelter veterinary staff. Samples from 32 of these cats were sequenced. Feces were collected when possible from the colon of FIP affected cats at the time of necropsy. Diseased tissues and feces were frozen at -40 to -70°C until used. Fecal samples from 184 healthy cats, usually kittens of neutering size and age, were obtained from litter boxes from June to October 2009. Forty five of these samples were tested and 31 (69%) found to be positive for coronavirus and 27/31 were sequenced.

2.2. Feline coronavirus nomenclature

FECV and FIPV are considered as biotypes of feline coronavirus (FCoV). For the purpose of this study, the original name FECV will be applied to coronavirus present in the feces of healthy cats (Pedersen et al., 1981) and FIPV to coronavirus present in diseased tissues or exudates of cats with necropsy confirmed FIP (Ward, 1970). Alternatively, the terms FCoV or “coronavirus” will be used when the disease potential of a particular isolate has not yet been tested by cat infection or when speaking about both biotypes of feline coronavirus in a generic manner.

2.3. Viral inocula

Diseased tissue, usually inflamed omentum or granulomatous lesions from kidney or mesenteric lymph nodes, was harvested at necropsy from cats that had died of FIP and stored at -70°C . Frozen tissue collected at necropsy was ground to a paste using sterile sand in a mortar and pestle. The ground material was then suspended in 0.25 g/ml HBSS, mixed thoroughly by vortex and centrifuged for 20 min at $2000 \times g$. The supernatant was retained and used for animal inoculation studies. Each inoculum was aliquoted and stored at -70°C . Feces were suspended at 0.5 g/ml in HBSS containing 7% FBS and thoroughly mixed while kept on ice. The suspension was centrifuged at $2000 \times g$ for 20 min to remove coarse material, and then at $8000 \times g$ for 10 min to remove finer debris. The resultant supernatant was aliquoted and kept frozen at -70°C .

The viral inocula used in the study are described in Table 1. The FECV-1 inoculum was made from pooled feces of healthy shelter cats SFS-25, SFS-26, SFS-27, and SFS-31. FCoV-1 inoculum was made from the feces of SFS-05 at the time of its death from FIP. The i3c-1 inoculum was a mixture of FIP lesional coronavirus from SF-SPCA cats SFS-01, SFS-04, SFS-10, and SFS-16; all of these isolates possessed an intact 3c gene. The i3c-2 inoculum was a mixture of lesional coronavirus from laboratory cats 09-017, 09-024, and 09-027 that were infected with inoculum i3c-1. Inoculum i3c-2 (feces) was derived from feces collected days post infection (dpi) 19 from cats inoculated with i3c-2. The m3c-1 inoculum was a mixture of FIP lesional coronavirus from SF-SPCA cats SFS-02, SFS-03,

Table 2
Objectives of individual cat infection experiments.

Expt.	Inoculum	Objective
1A, 1B	FECV-1	To determine the infectivity of FECV and fecal shedding status by different routes (i.p. and oronasal).
2	FCov-2	To determine whether the fecal coronavirus shed by naturally occurred FIP cat can induce FIP in experimental cats by oronasal route.
3A, 3B	i3c-1, m3c-1	To confirm that both of the FIPV isolates containing either intact 3c or mutate 3c genes can induce FIP in experimental cats.
4A	i3c-2	To determine whether cats with previous exposure will resist FIPV challenge.
4B	m3c-1	To determine whether the FIPV with a mutated 3c will cause enteric infection and FIP when given by oronasal route.
5A	i3c-2	To determine whether the FIPV with an intact 3c will cause enteric infection and FIP when given by oronasal route.
5B	m3c-2	To confirm that FIPV with a mutated 3c will induce FIP, but not to cause enteric infection when given i.p.
6A, 6B	i3c-2, m3c-2	To confirm whether the FIPV with an intact or mutated 3c will cause enteric infection and FIP when given by oronasal route.
7B, 8B	ddFIPV (tissue)	To determine whether the purified ddFIPV isolate can cause enteric infection and FIP in experimental cats.
9A, 10A	i3c-2 (feces)	To determine whether the fecal coronavirus shed by experimental FIP cats can induce enteric infection and FIP by i.p. or oronasal route.
9B, 10B	ddFIPV (feces)	To determine whether the fecal coronavirus shed by experimental FIP cats can cause enteric infection and FIP in experimental cats infected i.p. or oronasal.

SFS-08, and SFS-20; all isolates in this inoculum contained a mutated 3c. The m3c-1 inoculum also contained a virus designated ddFIPV as a minor variant; ddFIPV contained a double deletion in 3c and appeared as the dominant virus in experimental cat 09-029. The ddFIPV (feces) inoculum was made from dpi 20 feces of cat 09-029. The m3c-2 inoculum contained a lesional coronavirus from laboratory cat 09-022, which had been infected with m3c-1 by the i.p. route. This FIPV isolate possessed a mutated 3c.

2.4. Experimental animals

Specific pathogen free cats, ranging from 4 months to 4 years of age, were obtained from the breeding colony of the Feline Nutrition and Pet Care Center, University of California, Davis. Cats were housed in feline research facilities of the Center for Companion Animal Health, UC Davis.

2.5. Animal inoculation studies

Laboratory animal studies were conducted under Institutional Animal Care and Use Committee protocol #16637. Laboratory cats were infected either i.p. or oronasally (one-half orally, one-fourth in each nostril) with 0.5 ml of a cell free tissue extract of diseased tissues or an extract of feces. The various experiments and their objectives for these animals are given in Table 2. Rectal temperatures were recorded starting one to two days prior to inoculation and at 1–2 day intervals thereafter. Cats were examined daily for signs of disease such as fever, inappetence, depression, diarrhea, dehydration, ascites, hyperbilirubinuria, and jaundice. Once signs of FIP became apparent, usually 3–4 weeks after exposure, affected cats were euthanized with an intravenous overdose of pentobarbital/phenytoin and necropsies were performed to confirm their FIP status.

2.6. Purification and quantitation of feline coronavirus from feces

Fecal samples were suspended with 5 volumes of phosphate buffered saline (PBS) by vortexing. The suspension was centrifuged at $8667 \times g$ for 10 min and the supernatant transferred to a new tube and centrifuged at $54,174 \times g$ for 30 min. The pellet containing the virus was suspended with 5 ml PBS and centrifuged again at $54,174 \times g$ for 30 min. This pellet was suspended in 140 μ l PBS and the viral RNA extracted using a QIAamp Viral RNA mini kit (QIAGEN, USA). Briefly, 560 μ l lysis buffer containing carrier RNA was mixed with the 140 μ l viral suspension and incubated at ambient temperature for 10 min; 560 μ l 100% ethanol was added to the lysate. The lysate mixture was applied to QIAamp mini spin column and

the RNA binding to the column was achieved by centrifugation. The column was then washed and the RNA was eluted with 50 ml RNase-free water and stored at -70°C .

Feline coronavirus shedding was quantified using purification procedures and specific primers reported by Gut et al. (1999). RNA was extracted from feces by a modification of the procedure reported by van der Hoek et al. (1995) in order to exclude fecal inhibitors. Five microliters of the purified RNA was added to 7 μ l of PCR mixture containing 6 μ l of TaqMan One Step RT-Master Mix (Applied Biosystems, Foster City, CA), 0.31 μ l of MuLV/RNase Inhibitor, 0.24 μ l each of forward and reverse primers, and 0.10 μ l of RNase-free water. The 12 μ l reaction went through a reverse transcriptase step for 30 min at 48°C and AmpliTaq Gold (Applied Biosystems, Foster City, CA) activation for 10 min at 95°C . The samples were put through 40 cycles of 95°C for 15 s and 60°C for 60 s for RNA amplification. PCR was performed using Applied Biosystems (Foster City, CA) 7300 Real-time polymerase chain reaction (RT-PCR) System and 7300 System Software.

2.7. Isolation of viral RNA from diseased tissue

Viral RNA was extracted from omentum or granulomatous lesions on internal organs using QIAgenRAeasy mini kit (QIAGEN, USA). About 30 mg tissues were freeze dried with liquid nitrogen, ground with a mortar and pestle, and lysed with 600 μ l lysis buffer containing β -mercaptoethanol. After thoroughly mixing, the lysate was homogenized with QIAshredder (QIAGEN, USA) and an equal volume of 70% ethanol was added to the homogenized lysate. The lysate mixture was applied to RNeasy spin column and the RNA binding to the column was achieved by centrifugation. The RNeasy spin column was then washed and the RNA was eluted with 50 μ l of RNase-free water and stored at -70°C .

2.8. Sequencing of feline coronavirus 3a–c, E, and M genes from tissue and fecal extracted RNA

The published sequences of feline coronaviruses in GenBank were used to design the primers for a reverse transcriptase polymerase chain reaction (RT-PCR). Primer pairs were designed from highly conserved regions and used to amplify a 2.4 kb fragment containing the structural (E and M) and three accessory (3a–c) genes of feline coronavirus (Table 3). The RT-PCR was carried out with QIAgenLongRange 2 Step RT-PCR kit (QIAGEN, USA). The viral RNA was first denatured by incubating at 65°C for 5 min and then chilled on ice. The reverse transcription was carried out in 20 μ l reaction mixture containing 10 units of LongRange reverse transcriptase, 0.8 unit of RNase inhibitor, 1 mM dNTP, 1 mM OligodT,

Table 3

Primer sequences used for the amplification of the 3a–c, E, and M genes of feline coronavirus isolates as defined by the genome of FIPV-WSU-79/1146 (NC.0007025).

Primer name	Nucleotide sequence	Genome position
Forward primer	5'-GGCCTTGGTATGTGTGGCTAC-3'	24380
Reverse primer	5'-CTATTCCAATAACCAATTGTGATC-3'	26996

and 5 µl of denatured viral RNA in 1× reaction buffer. The mixture was incubated at 42 °C for 2 h followed by 85 °C for 5 min. The reverse transcribed cDNA was stored at –20 °C or used immediately in PCR amplification. The viral cDNA was amplified in 20 µl reaction mixture containing 2 µl cDNA, 1 unit LongRange PCR enzyme mix, 0.5 mM dNTP, 0.25 mM forward primer, and 0.25 mM reverse primer in 1× PCR buffer. The mixture was then incubated at 93 °C for 3 min and amplified for 30 cycles at 93 °C for 30 s, 60 °C for 30 s, and 68 °C for 1 min per kb of PCR product, followed by a final extension for 10 min at 68 °C. The reverse transcribed viral RNA from feces was amplified for 40 cycles under the same condition. The PCR products were electrophoresed in TAE buffer on a 0.8% agarose gel. The PCR product was purified using a QIAgen gel purification kit (QIAgen, USA).

Purified PCR products were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in 15 µl reaction containing 1 µl Big Dye terminator mix, 2 µl reaction buffer (5×), 35 ng sequencing primer, and 3 µl (out of 50 µl) gel purified PCR product. The sequencing reaction was incubated at 93 °C for 2 min and then amplified for 40 cycles at 93 °C for 20 s, 50 °C for 20 s, and 60 °C for 4 min. Unincorporated dye terminators and dNTP were removed by gel filtration based Performa DTR Ultra 96-well plate kit (EdgeBio, USA) and the cycle amplified products were analyzed by capillary electrophoresis using an ABI 3730 Genetic Analyser (Applied Biosystems, USA). Vector NTI advance 10 software (Invitrogen, USA) was used for alignment of sequence data. The percent sequence identity for pairwise alignment and the phylogenetic relationship among different FIPV and FECV isolates was analyzed using ClustalW2II (www.ebi.ac.uk/tools/clustalw2/).

Sequence analysis often identified one major and one minor virus strain in a tissue or fecal extracts. In some situations, additional strains became evident upon analysis of material resulting from cat passage. When such strains were identified, strain specific PCR primers were used to identify the presence of that particular strain in the original fecal or tissue extracts. Although more than two related strains may have coexisted in a particular sample, cloning was not done because of its potential to generate a large number of sequence artifacts.

2.9. Feline coronavirus antibody tests

Antibody titers to feline coronavirus were determined by an indirect fluorescent antibody (IFA) procedure using FIPV-WSU-79-1146 infected CRfk cells (Pedersen, 1976).

3. Results

3.1. Genetic characterization of feline coronaviruses from feces of healthy shelter cats and from diseased tissues and feces of cats that died from FIP following adoption

3.1.1. Characterization of the 3c gene of coronavirus isolates from FIP diseased tissues (FIPVs)

Forty cats originating from the shelter died of FIP following their adoption in 2008, providing a minimal estimate of FIP incidence among adoptees of 40/3000 or 1.3%. Diseased tissues from 35/40 of these cats yielded potentially useable coronavirus RNA. Fecal

samples were available from only two of these cats; both tested positive for coronavirus by real time PCR, and one (SFS-05) was used for cat infection studies and the other (SFS-119) was sequenced. Forty five healthy cats present in the shelter from June to December 2009 were sampled and tested for fecal coronavirus shedding and 31/45 (69%) were positive by real time PCR. The E, M, 3a–c genes from 32/35 of the FIP viruses and 27/31 fecal isolates from healthy cats were then successfully sequenced.

Nineteen of 32 (59.38%) of the sequenced FIP isolates had functional mutations involving 3c (Fig. 1). Isolates from cats SFS-20 and -21 were identical, thus yielding 18 unique mutations. All these mutations involved the creation of premature stop codons (10/18) or frame shifts (8/18) causing truncation of the 3c product. Almost all of these mutations were unique to the cat from which it was obtained (Fig. 1). Four of 19 of these tissue extracts also contained an intact 3c variant (SFS-06, 15, 20, and 21). One of the 19 FIP isolates (SFS-11) had a mutation that abolished the stop/start codon at the 3b/3c junction and yielded an extended 3b protein and no 3c product (Fig. 1). This isolate also had two deletion mutations in 3c (Fig. 1). Similar deletion mutations occurring downstream of a premature stop codon were also observed in three other FIPV isolates (SFS-03, 14, and 23). One FIPV isolate (SFS-01) had a premature stop code in 3a, while isolate SFS-18 had a 3-nucleotide deletion in the M gene (Fig. 1). The remaining 11/32 (34.4%) FIP tissue isolates had intact 3c genes and normal sized transcripts for 3a, b, E, and M.

3.1.2. Characterization of the 3c gene of fecal coronavirus isolates from healthy and FIP shelter cats

No significant mutations in 3c other than amino acid substitutions were detected in 26/27 FECV isolates. An isolate from SFS-68 had a single histidine insertion at amino acid position 17 of the 3c protein (Fig. 2). Virus was detected in the feces of two cats that died of FIP. The fecal virus from SFS-119 had the same 3c mutation as the tissue virus. Fecal and tissue isolates from cat SFS-05 had an identical intact 3c and the fecal virus (FCoV-2) was used for cat passage studies (Expt. 2, Table 4).

3.2. Pathological (Biotype) characterization of tissue and fecal coronavirus isolates

Coronavirus isolates from the feces of 4 healthy shelter cats (SFS-25, 26, 27, and 31) behaved as FECVs when inoculated oronasally into laboratory cats (Expt. 1A, Table 4). Isolates from SFS-27 and 31 were genetically different, while isolates from SFS-25 and 26 were identical to each other but different from SFS-27 and 31 (Fig. 3). Virus appeared in the feces within 2–4 days and was still being shed at 154 days post infection (dpi) (Expt. 1A, Table 4, Fig. 4a). Only one FECV variant among three variants in the pooled inoculum was detected in the feces during this period. This variant was traced to feces from cats SFS-25/26. The coronavirus shed in feces from day 4 to 30 was 100% identical to the inoculated virus, while the virus detected at day 154 had acquired 2 nucleotide (nt) substitutions (C to A and a T to C) changing threonine to asparagine and leucine to serine at amino acid positions 26 and 28 of the M protein. The same inoculum was then given by the i.p. route (Expt. 1B, Table 4). One out of 3 cats shed coronavirus in the feces and seroconverted. This fecal virus was identical genetically to the virus detected in the feces of cats that were infected oronasally with the same inoculum. This experiment confirmed that FECVs can infect the gut via the i.p. route, but with some difficulty.

Sixteen of 22 (73%) cats developed FIP after being infected oronasally or i.p. with inocula containing intact 3c virus, whether from naturally (Expt. 3A) or experimentally infected cases (Expts. 4A–6A, Table 4). Cats infected oronasally with intact 3c virus shed coronavirus in the feces (Expts. 5A, 6A, Table 4; Fig. 4b). Although coronavirus was detected in feces after the first passage in

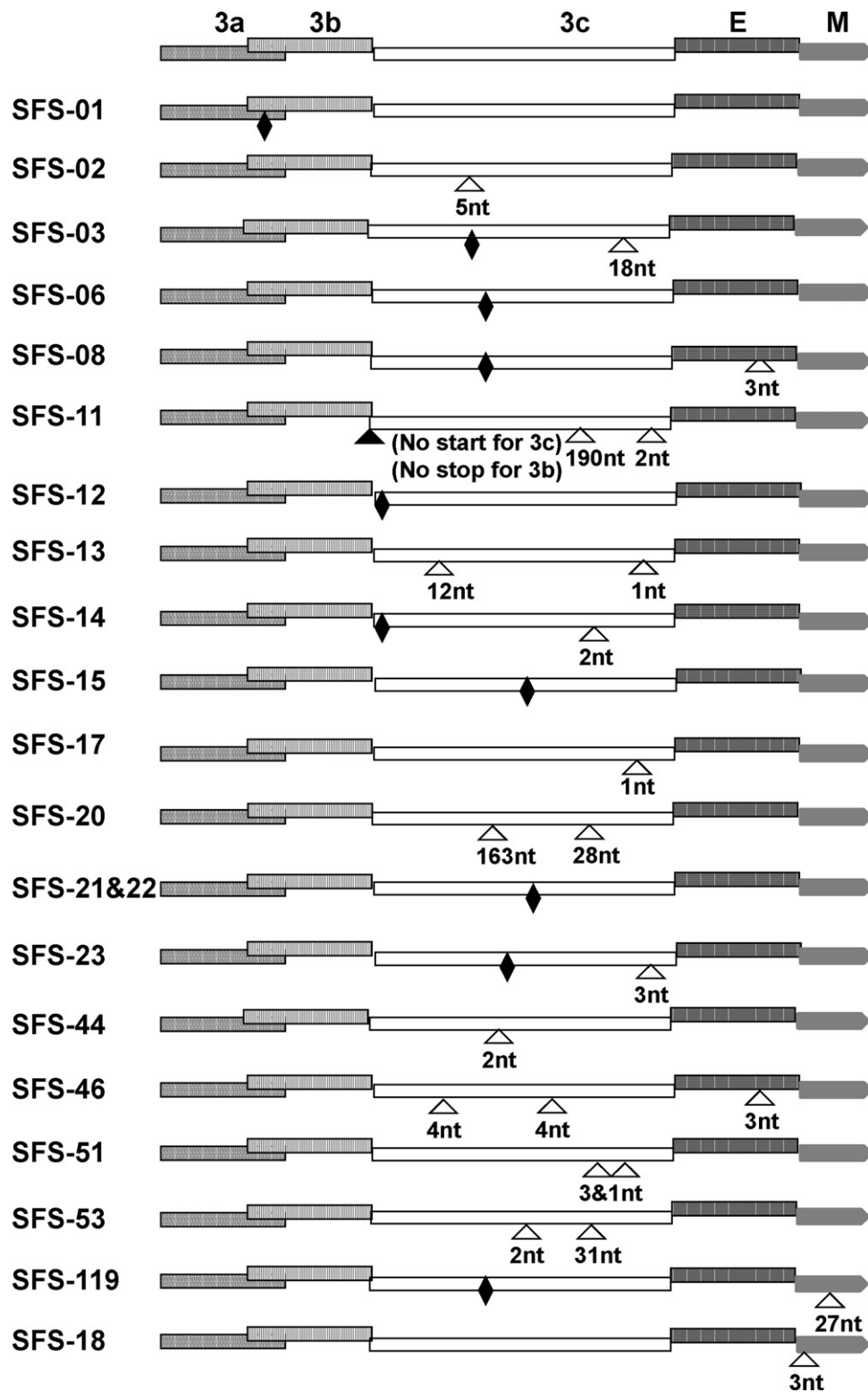


Fig. 1. Schematic representation of 3a–c, E, and M genes of 21 of 32 lesional isolates from cats dying of FIP after being adopted from the SF SPCA shelter during 2008. Nineteen isolates have functional mutations in 3c, and two have intact 3c genes, but with nt substitution creating stop codon in 3a (SF-01) and a 3nt deletion in M (SF-18). The remaining 11 isolates with intact 3c had no functional mutations in any of these genes and were therefore not illustrated. ♦, nucleotide (nt) substitution creating stop codon. Δ, deletion mutation and # of nt deleted.

laboratory cats, the virus was not infectious upon further cat passage by either oronasal or i.p. routes (Expts. 9A, 10A, Table 4). Coronavirus positive feces from one cat (SFS-05) that died of naturally acquired FIP was also not infectious when tested by the oronasal route (Expt. 2, Table 4).

Twenty nine of 37 (78%) cats developed FIP following oronasal or i.p. infection with FIP isolates possessing functional 3c mutations (Expts. 3B–8B, Table 4). Fecal virus shedding was not detected in cats from these experiments, with one exception. The exception occurred among cats infected oronasally with inoculum m3c-1 in

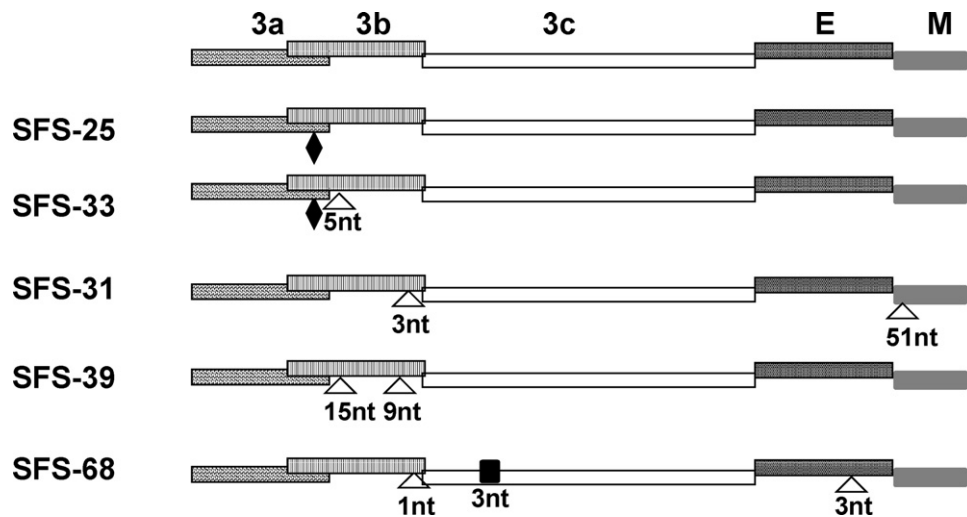


Fig. 2. Schematic representation of nonsense and deletion/insertion mutations in 3a–c, E, and M genes of FECV isolates from SF-SPCA shelter in 2009. ♦, nonsense mutations creating premature stop codons. Δ, deletion mutations with #nt deleted. ■, insertion mutation with #nt inserted.

Table 4
Results of experimental infection studies.

Expt.	FCoV	3c status	Isolate	Route	# cats	# FIP	Fecal shedding	Sero-conversion	Shedding period (days)
1A	FECV	Intact	FECV-1	Oral	3	0	3/3	Positive	154
1B	FECV	Intact	FECV-1	i.p.	3	0	1/3	Positive	25
2	FCoV	Unknown	FCoV-2	Oral	2	0	0/2	Negative	28
3A	FIPV	Intact	i3c-1	i.p.	7	7	ND	ND	ND
4A	FIPV	Intact	i3c-2	i.p.	8	4	0/8	ND	41
5A	FIPV	Intact	i3c-2	Oral	3	2	3/3	ND	61
6A	FIPV	Intact	i3c-2	Oral	4	3	4/4	Positive	95
3B	FIPV	Mutated	m3c-1	i.p.	7	7	ND	ND	ND
4B	FIPV	Mutated	m3c-1	Oral	6	6	6/6	ND	21
5B	FIPV	Mutated	m3c-2	i.p.	11	7	0/11	ND	21
6B	FIPV	Mutated	m3c-2	Oral	4	2	0/4	ND	60
7B	FIPV	Mutated	ddFIPV (tissue)	i.p.	7	7	0/7	ND	34
8B	FIPV	Mutated	ddFIPV (tissue)	Oral	2	0	0/2	Negative	34
9A	FCoV	Intact	i3c-2 (feces)	i.p.	4	0	0/4	Positive	45
10A	FCoV	Intact	i3c-2 (feces)	Oral	6	0	0/6	Negative	41
9B	FCoV	Mutated	ddFIPV (feces)	i.p.	3	0	0/3	ND	34
10B	FCoV	Mutated	ddFIPV (feces)	Oral	2	0	2/2	Positive	4, 7, 154 ^a

^a Cats only shed the FCoV/ddFIPV fecal virus through dpi 7. An FECV isolate shed by another cat in an adjacent room was detected with ddFIPV (feces) on dpi 11 and became the sole isolate thereafter.

experiment 4B (Table 4). Six out of six infected cats developed FIP and shed virus in their feces. An identical double deletion 3c mutant was isolated from tissues (ddFIPV-tissue) and feces (ddFIPV-feces) of all six cats. This mutant, which was not present at detectable levels in the m3c-1 inoculum, was traced back to the diseased tissues of cat SFS-20 using ddFIPV specific PCR. Tissue from SFS-20 was subsequently found to contain two variants. One variant had an intact 3c and one had a double deletion mutation in 3c (ddFIPV). ddFIPV (tissue) caused FIP in 7/7 cats by the i.p. route (Expt. 7B, Table 4) and in none of two cats given the inoculum orally (Expt. 8B, Table 4). No fecal shedding was detected when ddFIPV(tissue) was the sole virus in the inoculum (Expt. 8B, Table 4). The ddFIPV(feces) isolate did not cause FIP in cats by either oral or i.p. routes, but was briefly shed (days 4–7) following oral infection (Expts. 9B, 10B; Table 4). It was replaced in the feces by FECV-1 sometime between dpi 7 and 30 as a result of an inadvertent exposure to a cat in Expt. 1B (Table 4).

3.3. Variant feline coronavirus forms in diseased tissues and feces

3.3.1. Naturally infected cats

Mutational events in the 3a–c, E, and M genes of variants in the same tissue (FIPVs) or fecal sample (FECVs) were compared

(Table 5). A greater proportion of FIPV isolates contained mutations than FECV isolates (34.4% vs. 11.1%, respectively), and the number of mutational events per isolate was also greater for FIPV than FECV isolates (Table 5).

3.3.2. Experimentally infected cats

The infectious inocula used in this study were made from diseased tissues pooled from several cats. This was done for two reasons: (1) to test the disease potential of a greater number of coronavirus isolates using fewer laboratory cats and (2) to study the relative infectivity of different strains. Care was taken that each mixture contained only variants with either intact 3c or mutated

Table 5
Number of mutational events (SNPs, insertions, deletions) detected in the 2.4kb fragment between variants present within the same tissues (FIPVs) or feces (FECVs) of naturally infected cats.

	# of mutational events					% isolates with mutational events
	1	3	4	5	≥7	
FIPV	5	2	1	1	2	11/32 (34.4%)
FECV	2	1	0	0	0	3/27 (11.1%)

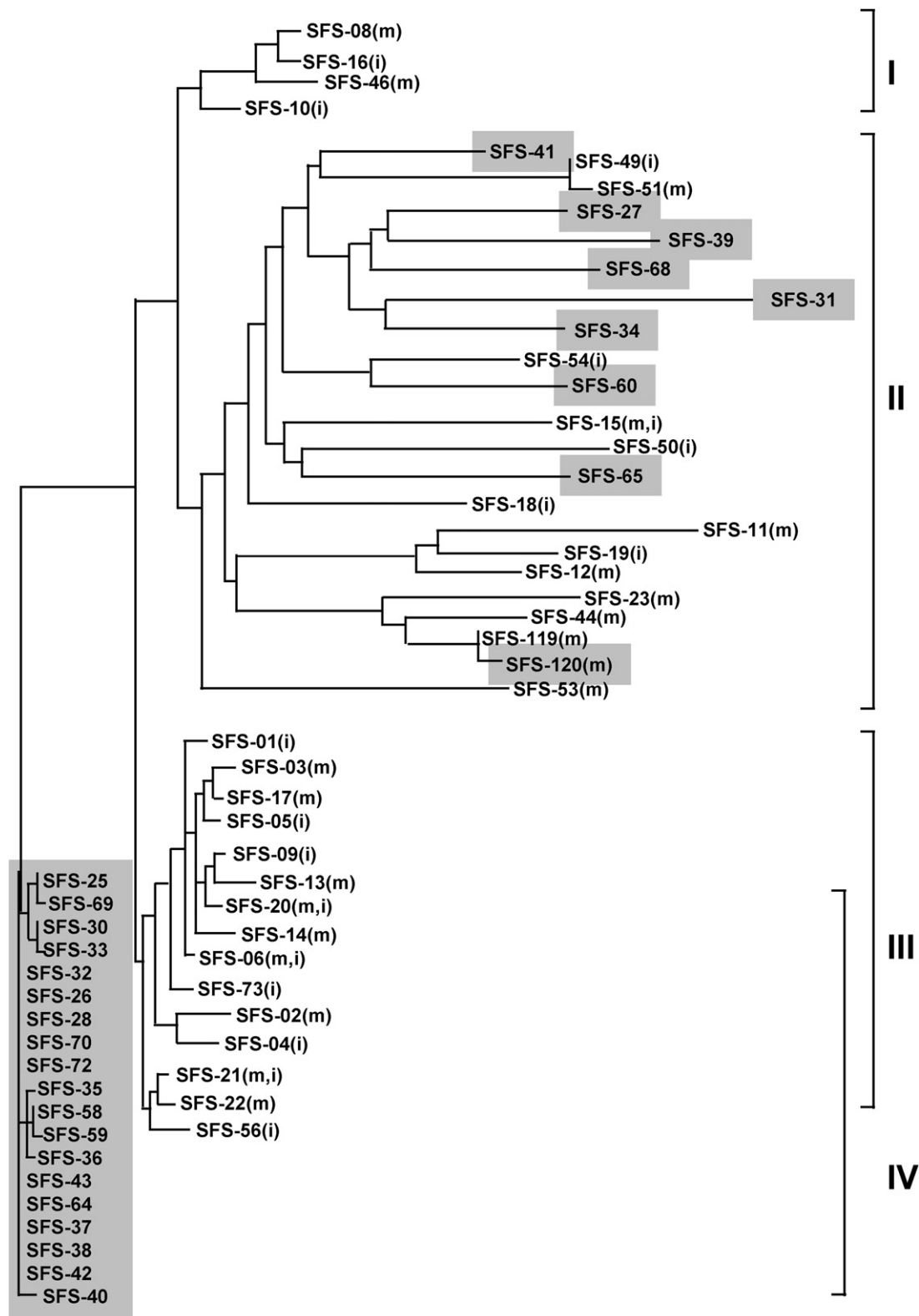


Fig. 3. Phylogram of feline coronavirus isolates from the SF SPCA shelter in 2008–2009. Twenty seven isolates were from the feces of healthy cats (shaded) and 32 were obtained from FIP lesional tissues (non-shaded). The fecal coronavirus isolate SF-120 was from FIP cat SFS-119.

3c. Following infection with a mixture of isolates, only one or two strains were associated with disease and the infecting strain(s) often varied from cat to cat among an experimental group (Fig. 5). The infecting strain(s) were either identical to the strain(s) found in the inoculum or a recombinant. For example, one of the infecting

variants in Cat-025 and Cat-022 was a recombinant between two isolates that were present in the infecting material (Fig. 5). The predominant infecting strain could also possess mutations not detected in the parent virus (Cat-007, 016, 025, 026, 027, Fig. 5a). In one study (Expt. 3B), which used an inoculum (m3c-1) made

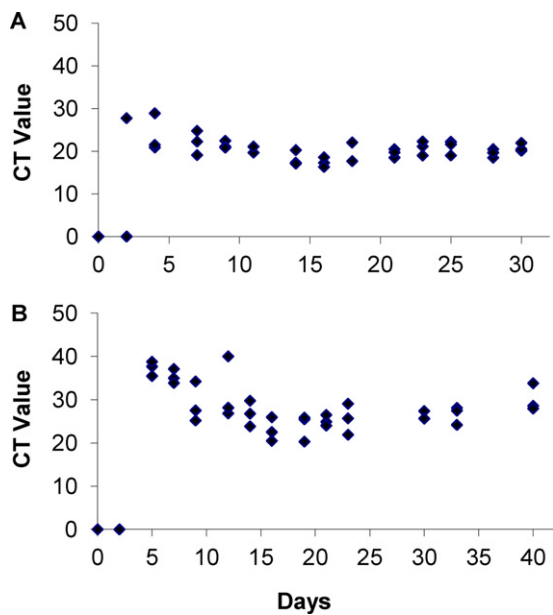


Fig. 4. (A) Fecal shedding of coronavirus from cats infected orally with FECV-1 (Expt. 1A). (B) Fecal shedding of coronavirus from cats infected orally with i3mc-2 (Expt. 6A).

from diseased tissues of four cats and presumably contained four mutated 3c variants, the infecting virus in five of six cats was a double 3c deletion mutant (ddFIPV) not initially detected in the inoculum (Fig. 5b).

3.4. Comparisons of 3a, b, E, and M gene products of FECVs and FIPVs

This part of the study involved a more in depth comparison of 3a–b, E, and M gene products of all FECV and FIPV isolates. FECV and FIPV isolates from the shelter cats belonged solely to serotype 1 based on 3a sequence analysis (Pedersen et al., 2009). Unique mutations were found in genes other than 3c in a number of FECVs. An identical mutation in 3a was detected in 17/27 FECV isolates resulting in a truncated 3a protein with 62 amino acids (see SFS-25 in Fig. 2). There were four FECV isolates (SFS-33, 31, 39, and 68) that had deletion mutations in 3b resulting in truncation of its protein (Fig. 2). One FECV isolate (SFS-68) had a single amino acid deletion at amino acid position 53 in the E protein, identical to that found in the four FIPV isolates of clade I (Figs. 2 and 3). Isolate SFS-31 had a 51nt deletion resulting in the loss of 17 amino acids starting at position 3 of the M protein (Fig. 2).

Strain specific mutations were also found outside of 3c in cats with naturally occurring FIP. The four cats with FIP (SFS-08, 10, 16, and 46) making up clade I had an identical single amino acid deletion at position 53 of the E protein (Figs. 1 and 3). All but one (SFS-13) of the FIP tissue isolates making up clade III contained a double amino acid insertion (isoleucine-proline) in 3c at positions 42 and 43 (Fig. 6). SFS-13 had a four amino acid deletion in the region where the isoleucine-proline insertion occurred. An identical isoleucine-proline insertion in the same position was also present in the 3c of all FECV isolates from clade IV, supporting the close genetic relationship of clades III and IV. Two isolates had functional mutations causing a premature stop codon in 3a (SF-01) and a 3nt deletion in M (SFS-18), even though they had intact 3c genes.

The 3a, b, E, and M amino acid sequences from the 32 FIPVs were compared to those of 27 FECVs to examine whether a specific mutation(s) in any of these proteins might differentiate FIPVs from FECVs. Similar to the 3c product (Fig. 6), a number of hypervariable

regions were identified in the 3a, b, E, and M proteins. The same hypervariable regions were present in both FECVs and FIPVs and no single amino acid changes or indels in these proteins were unique to either FECVs or FIPVs.

3.5. Comparison of intact 3c sequence and gene product of FECV and FIPV isolates

Non-synonymous amino acid changes were examined in 13 representative FECVs (8 from clade II and 5 from clade IV) and 13 FIPVs with intact 3c genes (2 from clade I, 5 from clade II, 6 from clade III) (Fig. 6). The number and position of non-synonymous amino acid changes were nearly identical from position 1 to 140. However, unique non-synonymous amino acid substitutions were more frequent in FIPVs than FECVs from position 150 to the 3' end of the 3c protein (17 non-synonymous changes in FIPVs vs. 4 in the FECVs) (Fig. 6).

3.6. Genetic relationships of FIPVs and FECVs from cats housed in the same shelter either before or after adoption

The 3a–c, E, and M sequences of isolates from lesional tissues of cats that died of FIP during 2008 and from feces of healthy cats that were in the shelter in 2009 were used to construct a phylogenetic tree (Fig. 3). Four clades (I–IV) of coronavirus were identified among this group of cats. Isolates within clades I and III were 98–99% identical to each other, while all clade IV isolates were virtually identical (99–100%). Clades I, III, and IV were 96–97% identical to each other, while clade II was 87–94% identical to clades I, III, and IV. Isolates from clade II were much more diverse, showing an overall identity of 87–94% (Fig. 3). This degree of relatedness is comparable to that encountered among isolates from geographically disparate regions of the US (Pedersen et al., 2009), and suggests that strains within clade II came from cats originating over a wide geographic area. In contrast, the close relatedness of coronavirus strains within clades I, III, and especially IV, indicated that each originated from a common source. This common origin was supported by the presence of characteristic mutations in each of these three clades (see Section 3.5). Cats infected with clades I and III entered the shelter over a 6 month period in 2008, while clade IV isolates were collected over a 6 month period in 2009, again suggesting that these cats were infected from three different sources.

4. Discussion

4.1. Incidence of functional 3c mutations in FIP virus isolates

Thirty two cats from a large regional shelter died of FIP within several months of being adopted as pets. Forty percent of the FIP isolates from these cats were found to have an intact 3c and 60% had deletion or stop codon resulted from nucleotide substitutions that yielded a truncated gene product. This was in agreement with previous studies on FIP isolates from the Netherlands (Chang et al., 2010).

4.2. Role of the 3c gene of FECVs and FIPVs in intestinal tropism and fecal shedding

The remainder of this study was an extension of the experiments reported by Chang et al. (2010) on the role of the 3c gene, except that the biotypes of coronavirus isolates from both healthy and FIP diseased cats were confirmed by infection of laboratory cats. Animal inoculation studies confirmed the basic conclusions of Chang et al. (2010), but with some modifications. Their first conclusion was that an intact 3c was essential for intestinal replication. Indeed, all FECVs studied had intact 3c genes and were infectious by the oronasal

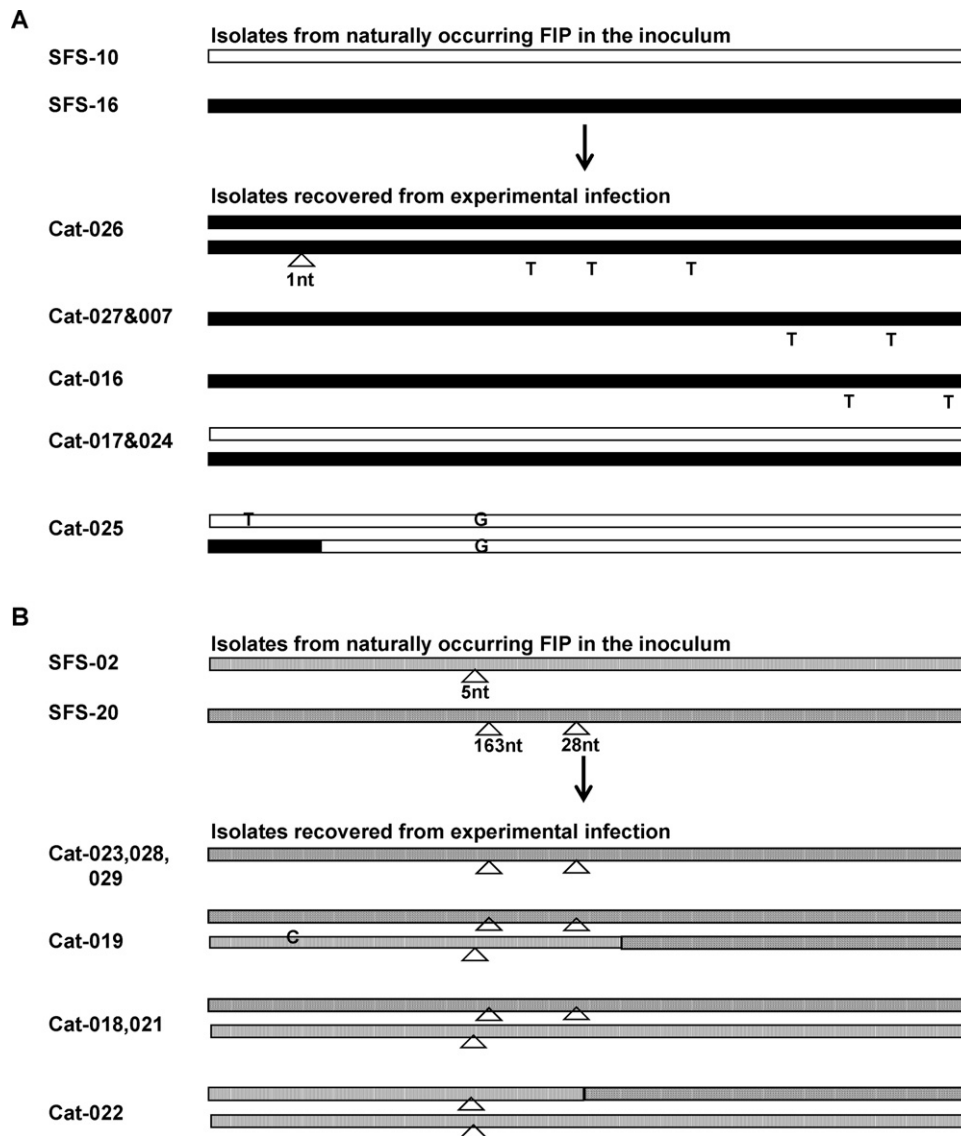


Fig. 5. Schematic representations of 3a–c, E, and M gene fragments of isolates detected in the lesional tissues of experimentally infected cats. SNP changes between original and cat passaged isolates are indicated by T, G, or C, while Δ indicates deletion of a single or multiple nucleotides (nt). (A) Coronavirus isolates from diseased tissues of cats experimentally infected with the i3c-1 inoculum (Expt. 3A). Isolates from these cats were either identical to those present in lesional tissues of cats SFS-10 and SFS-16 in the inoculum or recombinants of both strains. (B) Coronavirus isolates from diseased tissues of cats experimentally infected with inoculum m3c-1 (Expt. 3B). The isolates from these cats were either identical to those present in lesional tissues of cats SFS-02 and SFS-20 or recombinants of both strains.

route and shed at high levels in the feces. However, one of three cats infected i.p. with FECVs did shed virus from the feces, indicating that FECVs could reach the gut from the peritoneal cavity, but with some difficulty. Therefore, intestinal vs. macrophage tropism of FECVs may be relative rather than absolute. A second conclusion of Chang et al. (2010) was that mutations in 3c were not essential to cause FIP. Indeed, FIPVs with intact and functionally mutated 3c genes were equally capable of causing FIP when given by either oronasal or i.p. routes. Therefore, truncating mutations of 3c were not essential in causing FIP. Chang et al. (2010) also predicted that FIPVs with truncated 3c genes would not infect the intestine following oral infection and would not be shed in the feces. Although true for most isolates, there was again one exception. A 3c double deletion mutant (ddFIPV) was shed from the gut following oral infection with the m3c-1 inoculum. However, a fecal extract from laboratory cats shedding this particular mutant was not infectious for laboratory cats by the oronasal route. It is noteworthy that the original inoculum containing ddFIPV also contained a variant with

an intact 3c gene. This variant was no longer detected in feces after passage in laboratory cats. It is possible that ddFIPV used this variant as a helper for intestinal replication during its initial passage in laboratory cats.

The fecal shedding of FIPVs with intact 3c genes indicates the possibility of cat-to-cat (horizontal) transmission of FIP. The surprising finding in the present study was the lack of infectivity of coronaviruses that were shed in the feces of cats that were infected oronasally with FIPVs possessing an intact 3c. This lack of infectivity also applied to the novel double-deletion 3c mutant that appeared in the tissues and feces of cats infected with the m3c-1 inoculum and to a fecal virus from a cat with naturally acquired FIP that possessed the same intact 3c as the lesional virus. The reason for this lack of infectivity cannot be explained at this time, but is another example of the complexities of FIP pathogenesis. However, these findings support one corollary of the internal mutation theory, i.e., cats with FIP do not appear to effectively transmit FIP horizontally to other cats (Pedersen, 2009).

	10	20	30	40	50	60	70	80	90	100	110	120
SFS-01	MIGGLFLNTLGFIVTN	QHVIVNNTPHVDITVQQ	HHVVSANIPKSFLEFS	IAVLFLVFLALYRSTNFK	VCVGVLMFKIVMTLIG	PMLVAFGYIDIGVITTT	VLALFLFYLAYFWYVN					
SFS-04								L				
SFS-05												
SFS-73												
SFS-56										M		I
SFS-09												
SFS-10	S		NS									
SFS-16	S		NS									
SFS-18	S	VA	FHN	E	S	I				I		
SFS-49	S	V	NS	S					V	I		
SFS-19	S		NS	S			C	S		I		
SFS-54	S		NS				C		F	I		I
SFS-50	S		NS							I		
SFS-31	S		S	NSLT						I		A
SFS-39	S	V	S	A	S	G			V	I		
SFS-60	S	S	NS				I			I		
SFS-68	V	S	H	E	S	NS				I		
SFS-27	S		V	FQ	V					I		
SFS-41	S		NS	S						I		
SFS-34	S		NA	T	S					I		
SFS-33												
SFS-35												
SFS-40												
SFS-59												
SFS-65	S		N							I		A
SFS-69												

	130	140	150	160	170	180	190	200	210	220	230	240
SFS-01	SRFEFLINTTLTFVHDRA	APFMRSSHGSIYVTL	YGGINYMFDNLT	LHFDPLVGLIAIRGLVR	ADLTVVRAVELINGDF	IYIFSQEAVVGVYNAAS	QAVINEIDLKEEEER	VYDV				
SFS-04										F		
SFS-05										F		
SFS-73	H		F							H	F	
SFS-56			C							FF		
SFS-09					D					F		L
SFS-10								Y		F		G
SFS-16									V	F		G
SFS-18							H			F		C
SFS-49										F		A
SFS-19	V							V		F		Q
SFS-54										F		
SFS-50	V		L				H		T	F		Q
SFS-31		E								F		C
SFS-39										F		
SFS-60						V	H			F		
SFS-68										F		
SFS-27										F		
SFS-41							H			F		
SFS-34										F		
SFS-33										F		
SFS-35										F		
SFS-40										F		
SFS-59										F		
SFS-65	K									F		D
SFS-69										F		

Fig. 6. Amino acid sequence of the 3c protein of 13 FIPVs with intact 3c genes (shaded) and 13 FECVs (unshaded). Only non-synonymous amino acid changes are shown. Two FIPV isolates were selected from clade I, five from clade II, and six from clade III. Eight FECV isolates were selected from clade II, and five from clade IV. Isolates within each clade were chosen to be as unrelated to each other as possible.

4.3. Macrophage tropism of FECVs and FIPVs

It is generally assumed that FECVs are tropic for the mature intestinal epithelium, while FIPVs are tropic for macrophages. This implies that FECV infection is entirely contained within the intestinal tract. However, several studies have shown that FECV infection has both an intestinal and a systemic phase, and that the systemic phase involves monocyte-macrophages (Kipar et al., 2010; Vogel et al., 2010). This again suggests that intestinal and monocyte-macrophage tropisms of FECVs and FIPVs are relative and not absolute. This would explain how FECV was able to reach the intestinal epithelium in one of three cats infected with FECV by the i.p. route. If FECVs can replicate briefly in macrophage/monocytes, then the mutational transformation of an FECV to an FIPV may actually occur in monocyte-macrophages rather than intestinal epithelial cells. Such a scenario takes into account the high mutability of FECV and FIPV both within and between cats (Pedersen et al., 2009). The mutation rate may also be greater during replication in macrophages than intestinal epithelial cells, as indicated by the present study. High mutability would provide ample opportunity for positive selection for macrophage tropism. The better and longer an FECV can survive in macrophages following primary or secondary infections, the more adapted to macrophage growth it would become and the more virulent in terms of FIP. Indeed, Stoddart and Scott (1989) demonstrated that the ability

of an FIPV to replicate in macrophages increased with its virulence for cats. Mutational events (SNPs, insertions, deletions) in the present study were also found to be much more common in FIPVs than FECVs both in natural and experimental infections, indicating that FIPVs are under much more mutational pressure than FECVs.

The role of antibodies in FIPV infection and replication in macrophages needs to be considered as well. The timing of acquisition of serum antibodies in experimental FIPV, either by passive or active immunization, always coincides with the onset of disease (Pedersen and Boyle, 1980). Antibodies greatly enhance the uptake and replication of coronavirus in macrophages and contribute to subsequent disease signs through an arthus type reaction (Jacobse-Geels et al., 1982; Olsen et al., 1992; Pedersen and Boyle, 1980; Pedersen, 1986; Weiss and Scott, 1981). FIPV specific antibodies allow the mutant coronavirus an alternative pathway to enter macrophages, i.e., by Fc receptors. Therefore, the fate of the host cat may be linked to its ability to contain primary FECV replication in monocyte-macrophages before antibodies appear. In the present study, as with other studies of experimental FIP infection (reviewed Pedersen, 2009), a proportion of cats appear to resist experimental FIPV infection from the onset. The importance of an intact immune system in this initial containment has been demonstrated in a previous study using FECV infection of aged FIV infected cats and their non-infected siblings (Poland et al., 1996). There also

appears to be a genetic component to FIP susceptibility (Foley and Pedersen, 1996).

4.4. What is the mutation that causes FECVs to become FIPVs?

Chang and colleagues (2010) concluded that mutations in genes other than 3c can also lead to the FIPV biotype. However, they could find no evidence for such mutations. A comparison 3a–c, E, and M nucleotide and amino acid sequences of FECVs and FIPVs in the present study also failed to find any consistent differences in the 3a, 3b, E, and M gene sequences or products that would implicate these genes in FIP. One FIPV isolate (SFS-01) in the present study had a premature stop codon in the region overlapping 3a, b, another (SFS-11) had a mutation that resulted in an extended 3b protein and no 3c product, and two FIPV isolates had 3 nt and 27 nt deletions in the M protein. The 7a and 7b genes and their products were not studied herein, because isolates of both FECVs and FIPVs with significant mutations have been found to coexist in nature (Kennedy et al., 2001; Lin et al., 2009). Furthermore, deletion of 7b causes FIPVs to become avirulent (Herrewegh et al., 1995). Finally, significant mutations in 7a and 7b were not observed in a previous comparison of FIPVs and FECVs (Pedersen et al., 2009). Previous studies have also failed to implicate the spike (S) and nucleocapsid (N) genes in the FECV to FIPV biotype conversion (Battilani et al., 2010; Vennema et al., 1998). It is also possible that alternative FIP causing mutations may exist in the first two open reading frames comprising the replicase genes, which collectively make up more than two-thirds of the genome. However, the replicase tends to be highly conserved and mutations in these genes would be expected to inhibit virus replication and decrease virulence.

Although a singular importance of 3c mutations in the evolution of FIPVs has been questioned (Chang et al., 2010), such a conclusion may be premature in light of what is now known about variants. Although 60% of FIPVs have a functionally mutated 3c, such mutants often coexist with intact 3c variants in the same lesions. The opposite situation is also true in some cats. The pressure to progressively truncate 3c is also evident upon experimental cat passage (Pedersen et al., 2009). These observations suggest that mutations ultimately causing FIP do not start with the acquisition of premature stop codons, or frame shifting deletions and insertions that alter the size of the 3c product. FIPV causing isolates with intact 3c genes in the present study had four times more unique non-synonymous amino acid mutations in the 3' one-third of their 3c protein than FECVs. Although, Chang et al. (2010) reported no differences between 3c of their FECVs and FIPVs with intact 3c, a comparison of their FECV and FIPV 3c gene sequences obtained from GenBank also showed a similar trend from amino acid positions 160 to 240 of the 3c protein. The number of predicted non-synonymous amino acid substitutions in this region in 26 of their FECVs was 0.65/isolate compared to 2.0/isolate for 10 of their FIPV isolates. However, these differences may reflect a higher replication rate in macrophages vs. intestinal epithelial cells and play no role in FIP virulence. The ultimate answer as to the nature of FIP causing mutations may require, as suggested by Chang et al. (2011), the creation of infectious molecular clones of the basic feline enteric coronavirus by forward or reverse genetic manipulations, site directed mutagenesis to mimic various genotypes, and animal inoculation studies to confirm the resultant phenotype. Unfortunately, a true FECV has not been adapted to cell culture and most FIPVs also cannot be grown in vitro and the essential reagents are still missing.

4.5. Origins of FIP causing coronaviruses in shelters

Phylogenetic data from the large regional shelter sampled in this study allowed at least three conclusions: (1) feline coronavirus isolates within the shelter belonged to different genetically

defined clades, (2) clades differed genetically over time, (3) certain groups of cats in the shelter were infected by genetically identical FCoV, indicating a point source of infection occurring either prior to or during sheltering, and (4) not all FIP isolates could be traced back to the adopting shelter. These observations highlight essential differences in the dynamics of coronavirus infection in shelters vs. catteries or stable multi-cat environments. Catteries and stable multi-cat environments usually have one major enzootic strain of coronavirus that persists over long periods of time (Addie et al., 2003; Herrewegh et al., 1997; Pedersen et al., 2009; Poland et al., 1996; Vennema et al., 1998), even in the face of exposure to other strains (Addie et al., 2003; Herrewegh et al., 1997). Most previous studies on the origin of FIP viruses have involved catteries and multi-cat environments, which explain why FIP viruses have been genetically linked to enteric coronaviruses in the same environments (Hickman et al., 1995; Pedersen et al., 2009; Poland et al., 1996; Vennema et al., 1998).

A failure to understand the dynamics of coronavirus infection in stable vs. unstable cat populations can lead to very different conclusions on the origins of FIP causing coronaviruses. Brown and colleagues (2009) also did a genetic comparison of coronavirus isolates from 48 healthy and 8 FIP affected cats from a comparable large regional shelter in the Northeast of the United States. They concluded that FIP virus isolates were genetically distinguishable from enteric type coronaviruses in the shelter based on combinations of five specific amino acids at positions 108, 120, 138, 163, and 199 in the M protein. Two of these sequences, YIVAL (I) or YIIAL (II), were purportedly found in all cats with FIP, while isolates from healthy coronavirus infected cats were HIIVI (III), HIIIVL (IV), HVIAL (V), or YVVAL (VI), and uncommonly YIVAL (I). In agreement with Chang et al. (2011), the present results confirmed that these particular amino acid sequences in M have no value in distinguishing FECVs from FIPVs. All but two of the 32 FIPV and 27 FECV isolates in the present study had YIVAL(I) in their M protein. One FIPV isolate (SFS-50) had threonine (T) instead of alanine (A) at amino acid position 163, and one FECV (SFS-39) had an isoleucine (I) instead of valine (V) at position 120 and a valine (V) instead of isoleucine (I) at position 138. An alternative explanation for the conclusion of Brown et al. (2009) was that FCoV populations in a shelter are changing continually depending on the age of cats at the time of acquisition and their prior environmental exposure history. In order to trace the origins of FIP viruses in cats that have passed through shelters, one must sample feces prior to the usual age of primary coronavirus infection (9–10 weeks of age; Pedersen et al., 2008) and in subsequent exposure situations preceding the development of FIP.

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